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Overproduction of the regulatory subunit of the cAMP-dependent protein kinase blocks the differentiation of *Dictyostelium discoideum*

Marie-Noëlle Simon, Donna Driscoll[1], Rupert Mutzel[2], Dominique Part, Jeffrey Williams[1] and Michel Véron

Unite de Biochimie Cellulaire, Institut Pasteur, 75724 Paris Cedex 15, France, [1]ICRF Clare Hall Laboratories, South Mimms, Potters Bar, Herts. EN6 3LD, UK and [2]Fakultät für Biologie, Universität Konstanz, 7750 Konstanz, FRG

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During the aggregation of *Dictyostelium discoideum* extracellular cAMP is known to act as a chemotractant and as an inducer of cellular differentiation. However, its intracellular role as a second messenger remains obscure. We have constructed a fusion gene consisting of the cDNA encoding the regulatory subunit (R) of the cAMP-dependent protein kinase fused to the promoter and N-terminal-proximal sequences of a *Dictyostelium* actin gene. Stable transformants, containing multiple copies of this gene, overproduce the R subunit which accumulates prematurely relative to the endogenous protein. These transformants fail to aggregate. Detailed analysis has shown that they are blocked at interphase, the period prior to aggregation, and that they are severely defective in most responses to cAMP including the induction of gene expression. Our observations suggest that intracellular cAMP acts, presumably by activation of the catalytic subunit of the cAMP-dependent protein kinase, to facilitate early development.

Key words: differentiation/gene expression/protein kinase/slime mold/transformation

Introduction

Many cellular processes are regulated by cAMP. In prokaryotes, cAMP controls gene expression by binding to the catabolic activator protein (CAP) which interacts directly with the DNA (de Crombrugghe *et al.*, 1984). In higher eukaryotes, cAMP has been shown not only to regulate the activity of enzymes of metabolic pathways (Krebs and Beavo, 1979), but also to participate in the control of gene expression (Roesler *et al.*, 1988). All the cAMP-dependent processes in eukaryotic cells appear to be mediated by cAMP-dependent protein kinases (cAK). These enzymes are oligomers composed of regulatory (R) and catalytic (C) subunits (Flockhart and Corbin, 1982). The inactive holoenzyme dissociates into active C subunits upon binding of cAMP to the R subunits. It has recently been demonstrated that the phosphorylating activity of the cAK is directly involved in cAMP-dependent gene expression in mammalian cells (Boney *et al.*, 1983; Waterman *et al.*, 1985; Clegg *et al.*, 1987; Grove *et al.*, 1987; Riabowol *et al.*, 1988).

Extracellular signalling by cAMP plays a dual role in the development of the lower eukaryote *Dictyostelium discoideum* since cAMP is both a chemotractant during aggregation and an effector of gene expression (reviewed in Williams *et al.*, 1986; Kessin, 1988). Cyclic AMP molecules are detected by cell surface receptors, which elicit a number of intracellular responses, including *de novo* synthesis of cAMP, Ca^{2+} release, cGMP synthesis and inositol triphosphate accumulation (reviewed in Janssens and Van Haastert, 1987; Gerisch, 1987). There is no direct evidence to show which of these molecules acts as intracellular messenger(s) for the expression of genes regulated via extracellular cAMP signals in *Dictyostelium*.

The cAMP-dependent protein kinase from *D.discoideum* is also composed of regulatory and catalytic subunits but the holoenzyme is an RC dimer, as opposed to the typical R_2C_2 structure of the cAKs from other sources (Mutzel *et al.*, 1987). Although the R subunit of the cAK is developmentally regulated showing a low level in vegetative cells and a strong increase at the time of aggregation (Leichtling *et al.*, 1984; de Gunzburg *et al.*, 1986), the exact role of the cAK in the control of *Dictyostelium* development is not known. Experiments using cAMP analogs specific for either the membrane cAMP receptor or the R subunit of the cAK have demonstrated the involvement of the cell surface cAMP receptor in the regulation of some cAMP-dependent genes (Schaap and van Driel, 1985; Haribabu and Dottin, 1986). Binding of extracellular cAMP to the cell membrane can result in an increase in the intracellular cAMP concentration through receptor-mediated activation of the adenylate cyclase (Dinauer *et al.*, 1980). Alternatively, receptor-mediated cAMP-dependent gene expression in *D.discoideum* might be under the control of other intracellular 'second messengers' as indicated by experiments in which the activation of adenylate cyclase was inhibited (Schaap *et al.*, 1986; Oyama and Blumberg, 1986; Gomer *et al.*, 1986), or the study of developmental mutants impaired in the signal transduction mechanism (Wurster and Bumann, 1981; Schaap *et al.*, 1986; Bozzaro *et al.*, 1987; Snaar-Jagalska and van Haastert, 1988; Mann *et al.*, 1988).

In order to assess the role of cAMP-dependent protein kinase in differentiation, we have transformed *Dictyostelium* cells with a vector carrying the coding sequence of the R subunit of the cAMP-dependent protein kinase. In this paper, we demonstrated that overproduction of the R subunit in *Dictyostelium* cells disrupts normal development.

Results and discussion

The cDNA clone 10.1 (Mutzel *et al.*, 1987) containing the entire coding sequence and the 5′ and 3′ non-coding regions of the *Dictyostelium* R subunit transcript, was cloned in a sense orientation into the *Hin*dIII restriction site of an expression vector carrying a *Dictyostelium* actin gene (Pears, 1987). This site is located near the 5′ end of the coding region of the *D.discoideum* actin 15 gene (Knecht *et al.*, 1986), and allows expression of cloned sequences under

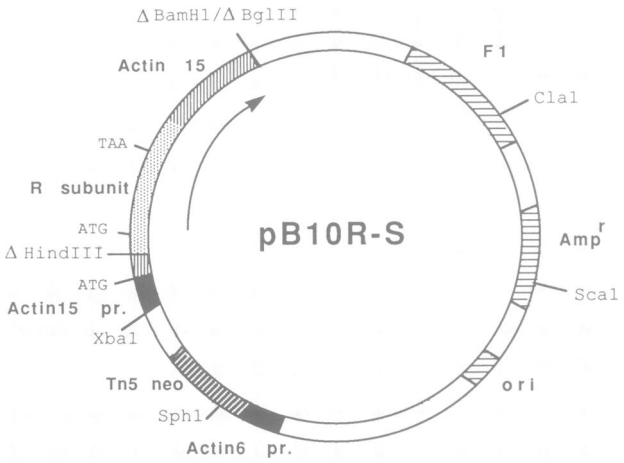

Fig. 1. Vector PB10R-S. The construction of the vector is described in Materials and methods. Promoters (pr.) of the actin 6 and actin 15 genes are drawn in black. Translation initiation codons (ATG) for actin 15 and R subunit sequences are shown, along with the TAA stop codon in the R subunit cDNA. Restriction sites that are not reconstituted after cloning are indicated by the sign Δ.

control of its promoter. Figure 1 shows the structure of the resulting construct, pB10R-S. *Dictyostelium* cells of the axenic strain Ax2, were transformed with pB10R-S. The pooled population of transformants were kept in liquid culture for several weeks under constant selection with the aminoglycoside drug G418. Southern blots probed with a cDNA corresponding to most of the coding sequence of the R subunit showed that the recombinant strains contained ~200 copies of the plasmid per cell (data not shown).

Transformant cells (4M cells), greatly overproduce R subunit mRNA and protein. Figure 2 shows an analysis of the accumulation of the R subunit mRNA in Ax2 control and in 4M cells at various times of development. In 4M transformants, a transcript of 3.4 kb is detected in vegetative cells and this increases in abundance by 3 h of development (Figure 2A). This transcript is of the size expected for a fusion mRNA containing both R subunit and actin sequences transcribed from the actin 15 promoter, and shows the pattern of accumulation expected for a transcript under control of the actin 15 promoter (Cohen *et al.*, 1986). It is readily resolved from the 2.2 kb RNA transcript of the endogenous R subunit gene (Figure 2B, lanes 3–15). The extent of overexpression of the R subunit mRNA is shown by comparing the hybridization signal obtained with total RNA from strain Ax2 lanes (3–15) and 4M (lane T). Slot blot analysis of various dilutions of RNA from Ax2 and 4M cells showed the level of the R transcript in 4M cells to be at least 50-fold higher than that of the endogenous R subunit mRNA (data not shown).

Transformants 4M also strongly overproduce the R subunit protein (Figure 3). Whereas in Ax2 cells the R subunit is only detectable on Western blots at 3 h, and gives a strong signal after 6 h, it is already highly expressed in vegetative 4M cells and shows a further increase upon starvation (Figure 3A). The amount of R subunit, monitored by measuring the cAMP binding activity in crude extracts, also showed a large increase in 4M as compared to Ax2 control cells (Figure 3B). Since the 5′ non-coding region of the R subunit cDNA contains stop codons in all three reading frames (Mutzel *et al.*, 1987), synthesis of the R subunit

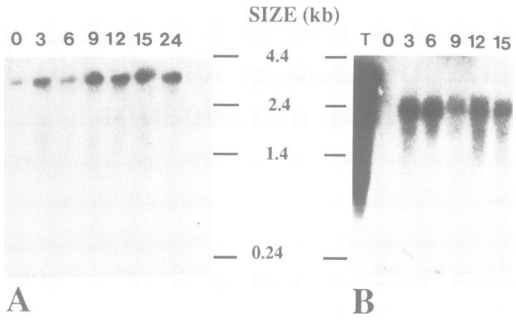

Fig. 2. Northern blot analysis of total RNA from Ax2 and 4M cells at various times of development. Total cellular RNA was extracted from cells allowed to differentiate on filters for the indicated times. 10 μg of total RNA were electrophoresed, blotted onto gene-screen-plus membranes as described in Materials and methods, and hybridized with the *Eco*RI–*Ava*II fragment of the cDNA coding for the R subunit (Mutzel *et al.*, 1987). (**A**) Analysis of RNA from 4M cells. 10 μg of total RNA from 4M transformants starved for various times were analyzed. The autoradiograph was exposed for 8 h. (**B**) Analysis of the RNA from Ax2 cells. 10 μg of total RNA from Ax2 cells starved for various times were analyzed. The film was exposed for 3 days in order to detect the endogenous mRNA for the R subunit in the parental Ax2 cells. A comparison with the level of mRNA shown in **A** is provided in lane T in which 10 μg of total RNA from transformant 4M starved for 15 h is analyzed. The presence of smaller transcripts in 4M cells (lane T) probably results from precocious termination of the transcription of the fusion gene in the poly(A) sequence at the 3′ end of the R-cDNA.

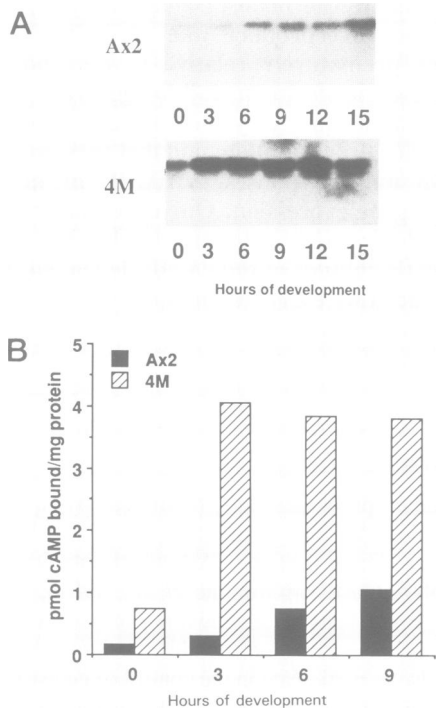

Fig. 3. Developmental regulation of the regulatory subunit in Ax2 and 4M cells. (**A**) Western blots of crude extracts from various stages of development. The samples (100 μg of total protein) were boiled for 2 min and subjected to electrophoresis in the presence of SDS. The gels were blotted onto nitrocellulose and the blots were reacted with anti-R antiserum, labeled with [^{125}I]protein-A and autoradiographed. In lanes where the amount of R subunit is very high, a faint band of lower mol. wt can be seen which is most likely due to proteolytic degradation, a phenomenon which has been previously observed for the R subunit protein (de Gunzburg *et al.*, 1984). (**B**) cAMP binding activity in crude cellular extracts. This was measured as described in Materials and methods. Solid bars: extracts from Ax2 cells. Dashed bars: extracts from 4M transformants. The results represent mean values of three different determinations.

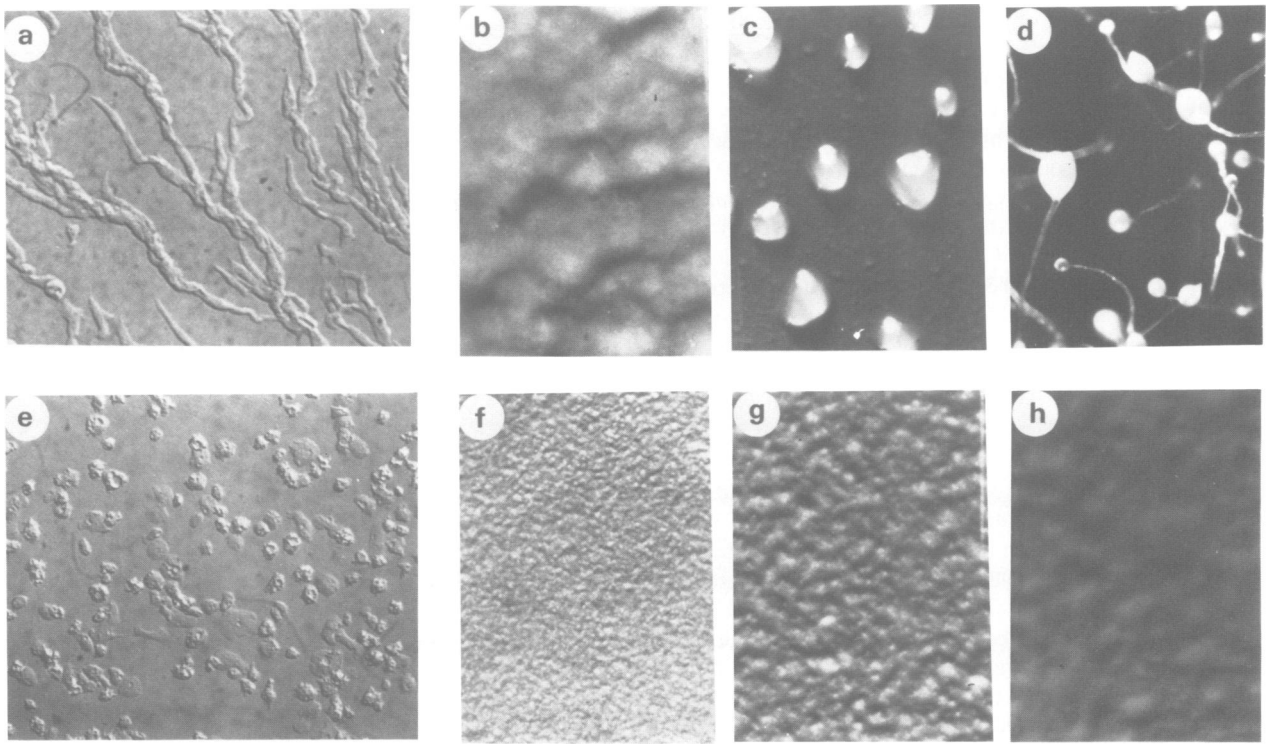

Fig. 4. Phenotype of transformant 4M: cells transformed with pB10R-S are blocked in aggregation. (**Panels a** and **e**) Differentiation under liquid immersion. Cells were grown in HL5 broth, washed and resuspended at 10^6 cells/ml in PB buffer (20 mM potassium-sodium phosphate buffer pH 6.2 containing 0.5 mM $CaCl_2$) and 1 ml was dispersed into small (3 cm diameter) tissue culture dishes. After 6 h of development at 22°C, the cells were observed with an inverted microscope. **a**: control Ax2 cells; **e**: cells from transformant 4M cells. (**Panels b–d** and **f–h**) Differentiation on filters. Cells were allowed to develop on filters at a density of 2×10^7 cells/filter as described in the legend to Figure 2 and the filters were photographed after various times of incubation at 22°C in the dark. **b, c** and **d**: control cells. **f, g** and **h**: transformant 4M. Photographs were taken after 3 h (**b** and **f**), 6 h (**c** and **g**), or 24 h of development (**d** and **h**).

protein must result from internal initiation of translation at the ATG of the R-cDNA. Hence the disparity between the extent of overproduction of protein (5- to 10-fold) and of the fusion RNA (50-fold) probably results from inefficient initiation of translation at this internal ATG.

We find the 4M transformants to arrest very early in development under a variety of experimental conditions. When deposited on filters in the absence of nutrients, wild-type cells proceed through the entire developmental cycle and form fruiting bodies but transformant cells fail to aggregate (Figure 4). When wild-type amoebae are spread on a cell culture dish and starved under buffer, the formation of aggregating streams of cells is readily visualized. Transformant amoebae starved in this way do not form streams (Figure 4). When control Ax2 cells are starved in suspension under constant agitation they form large aggregates and develop EDTA-resistant cell contacts after 6–7 h (Beug *et al.*, 1973) while 4 M amoebae form very small clumps and fail to form EDTA-stable cell contacts (data not shown). After transfer of these suspension-starved cells onto filters, small mounds of cells are formed but they do not proceed further through development. Finally, addition of cAMP in pulses to suspension starved cells, a method known to allow aggregation of a developmental mutant and accelerate wild-type differentiation (Darmon *et al.*, 1975; Gerisch *et al.*, 1975), has no effect on 4M cells, and does not induce the appearance of EDTA-stable cell contacts. 4M transformants were also analyzed for the expression of a developmentally regulated marker, the cysteine proteinase 1 gene (CP1) (Williams *et al.*, 1985). As shown in Figure 5A, there is no expression of the CP1 transcript in 4M cells

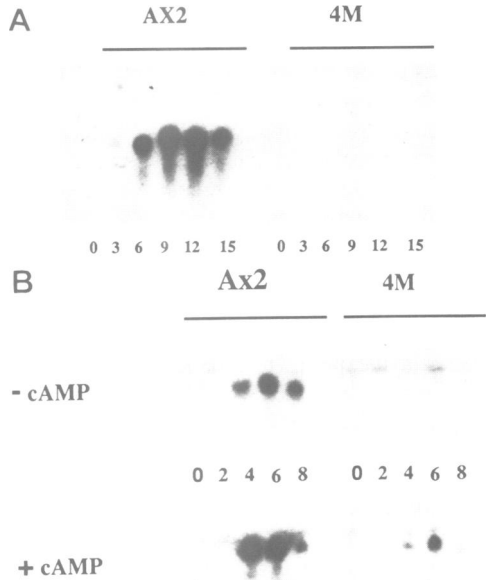

Fig. 5. Expression of CP1 mRNA transcripts in Ax2 and 4M cells. (**A**) Developmental regulation of CP1 mRNA accumulation. Ax2 and 4M cells were allowed to differentiate on filters and Northern blots were performed as described in Figure 2. The blots were probed with a mixture of the *Eco*RI–*Hin*dIII and *Bgl*II–*Sca*I restriction fragments from the coding sequence of the CP1 cDNA clone 11.7.10 (Williams *et al.*, 1985). (**B**) Induction of the CP1 mRNA by cAMP. Ax2 and 4M cells were starved in suspension at 10^7 cells/ml in PB buffer and shaken at 120 r.p.m. When indicated, 1 mM cAMP was added after 90 min, and aliquots of 10^7 cells were taken at the indicated times. Northern blots were performed as above and probed with the CP1 probe.

while in the Ax2 control the mRNA begins to accumulate at 6 h of development.

Despite this early block to differentiation, the cells retain a level of responsiveness to extracellular cAMP. When wild-type cells are placed at high cell density in a spectrophotometer cuvette, both the chemotaxis and the cAMP relay responses to extracellular cAMP signals can be monitored by a drop in light scattering of the cell suspension. The cAMP relay can be detected after 3 h of starvation and spontaneous oscillations appear somewhat later in development (Gerisch and Hess, 1974). Although 4M cells showed the relay response to exogenous cAMP as monitored by this technique (data not shown), they never produced autonomous oscillations even after prolonged starvation. Thus some of the early responses are retained in the mutant but from its phenotypic properties it appears to be blocked at a developmental stage equivalent to that reached by wild-type cells after ~3 h of starvation.

Several lines of evidence indicate that the overproduction of the regulatory subunit is the reason for the developmental defect in the transformant. The 4M cells were produced by pooling a very large number of independent transformant clones, hence it is exceedingly unlikely that insertion of the transforming DNA into a vital gene disrupted development. Analysis of a single clone picked from the transformed population showed no difference in overproduction of the R subunit and phenotype. Furthermore, in a separate experiment, transformant cells were cloned immediately after their appearance on the original transforming plate by inoculation of single cells into the wells of a microtitre plate. Seven such independently isolated clones were grown in the presence of increasing concentrations of G418. After selection for growth in 100 μg/ml of the drug all of the seven clones were totally aggregation defective. Finally, the block in differentiation in 4M cells is also not due to secondary effects of the presence of multiple copies of the vector because parallel transformation of Ax2 cells with another pB10−Act15 construct, and selection to high drug concentration, yielded transformants with multiple vector copies which formed normal fruiting bodies upon starvation.

Further evidence that R subunit overproduction causes the aberrant development derives from the observation that, when 4M cells are grown on a bacteria lawn and replated several times, they eventually develop fruiting bodies. In three independent experiments, the R subunit in 4M amoebae grown in this way was monitored by Western blots and by *in vitro* assay of [^{3}H]cAMP binding. In two cases the level of R subunit was the same as in Ax2 cells and in the third case it was found to be only twice that of untransformed cells (data not shown). There is no decrease in the plasmid copy number in cells grown on bacteria and spores collected from the resulting fruiting bodies germinate in HL5 broth in the presence of G418 to yield cells which are again aggregation defective, showing that the phenotypic reversion is not due to a loss of the vector. The reason for this shut-off of the overproduction of R-subunit when the cells are grown on bacteria is not clear at present. It is not due to differential expression of the actin 15 promoter, as was described for the actin 6 gene (Knecht and Loomis, 1987), because when coupled to a CAT reporter the level of expression is identical in bacterially and axenically grown cells (A.Harwood, personal communication). It may be due to a decreased efficiency of the reinitiation of translation leading to the synthesis of R subunit from the fusion transcript, or to differential stability of the mRNA or protein when grown on bacteria.

Overproduction of the R subunit presumably results in repression of the kinase activity of the catalytic subunit. Although the measured intracellular cAMP concentration in aggregating amoebae oscillates between 1 and 10−20 μM (Gerisch and Wick, 1975;Brenner, 1978), the free cytoplasmic cAMP concentration is possibly lower than this because the bulk of the cAMP may be sequestered in vesicles destined for release from the cell (Maeda and Gerisch, 1977). Consistent with such an hypothesis, the cAMP-dependent protein kinase is fully activated *in vitro* in the presence of 0.1 μM cAMP (Majerfeld *et al.*, 1984). Hence the enzyme would be constitutively activated if the free intracellular cAMP concentration was indeed in the micromolar range. Assuming a cell volume of ~8 μl/10^7 cells (Gerisch and Wick, 1975), and given the cAMP binding activity measured in cell extracts (Figure 3B), the intracellular concentration of R subunit in 4M cells should be ~1 μM. Thus 4M cells appears to synthesize enough R subunit to trigger a significant decrease in the free intracellular cAMP concentration, resulting in permanent inhibition of the catalytic subunit. One would expect that addition of cAMP to starving transformant cells could overcome this inhibition by eliciting a rise in the intracellular cAMP concentration. Consistent with this, we find that the CP1 mRNA is inducible when cAMP is added to a concentration of 1 mM to cells starving in suspension, although the level of accumulation is much lower than in control Ax2 cells (Figure 5B).

The results described here provide the first direct indication for an intracellular role of cAMP in the control of *Dictyostelium* development and we speculate that its action is mediated by the cAMP-dependent protein kinase. Experiments are under way to demonstrate this directly by altering the catalytic activity of the enzyme *in vivo*. Possibly, the inability of overexpressing transformants to progress beyond the pre-aggregative stage of development results from the absence of a cAMP-stimulated gene product, or products, required to enter the next phase of development. This mutant provides a start point for the isolation of these genes.

Materials and methods

Construction of transformation vector pB10R-S

The pB10R-S construct (see Figure 1) is derived from the vector pB10TP1 (Early and Williams, 1987). The *Xba*I−*Bgl*II restriction fragment from clone SC79 carrying the *Dictyostelium* actin 15 gene (Knecht *et al.*, 1986) was ligated into pB10TP1 which was double digested with *Xba*I and *Bam*HI, to yield pB10Act15 (Pears, 1987). A cDNA encoding the complete R subunit and also containing both 5′ and 3′ untranslated sequences (clone 10.1, see Mutzel *et al.*, 1987), was cloned into the blunt-ended single *Hin*dIII restriction site located in the actin 15 coding sequence, 19 bp downstream from the ATG initiation codon. The construct carrying the R-cDNA in a sense orientation relative to the actin 15 promoter was selected by restriction analysis.

Transformation of Dictyostelium discoideum

Transformation of Ax2 cells was carried out using the calcium phosphate procedure as described (Nellen *et al.*, 1984; Early and Williams, 1987). Usually, the population of transformant cells (~200 clones per plate) was grown as a pool, in the presence of increasing G418 concentrations, and kept in culture with constant selection for several weeks. When the transformed cells were to be cloned, single cells from the pooled population were used to inoculate 96-well microtitre plates containing 10 μg/ml of G418 in HL5 broth. After growth, the cells were transferred to flasks and the G418 concentration was progressively raised to 100 μg/ml.

RNA analysis

Cells were grown to 4–6 × 10^6 cells/ml in HL5 broth (Watts and Ashworth, 1970) supplemented with 100 μg/ml of G418 in the case of 4M cells. After harvesting, the cells were resuspended at 5 × 10^7 cells/ml in DB buffer (40 mM potassium phosphate buffer pH 6.2 containing 20 mM KCl, 2.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$) and 1 ml of cell suspension was deposited on black 47 mm nitrocellulose filters (Millipore) supported by a pad saturated with 1 ml of DB buffer. The filters were incubated in a humid atmosphere at 22°C and at the indicated times, 10^7 cells were collected and stored as pellets at −80°C. Total cellular RNA was prepared by the method of Alton and Lodish (1977). Aliquots of the RNA (10 μg per lane) were size-fractionated on 1% agarose–formaldehyde gels, blotted onto gene-screen-plus membranes in 10 × SSC (1.5 M NaCl, 0.15 M sodium citrate) and baked for 2 h at 80°C. The hybridization probes were prepared using the multiprime DNA labeling system (Amersham). After washing, filters were dried and autoradiographed using Kodak X-OMAT AR films with an intensifying screen.

Western blots

Cells were allowed to differentiate on filters as described above. The frozen pellets were resuspended in hot (90°C) gel sample buffer (Laemmli, 1970) and boiled for 2 min. 100 μg of total protein from each extract was subjected to electrophoresis in the presence of 0.1% SDS. Proteins were blotted from the gels to nitrocellulose by the method of Towbin *et al.* (1979) as modified by Khyse-Andersen (1984). The blots were reacted with anti-R antiserum, labelled with [^{125}I]protein-A and autoradiographed as described previously (Part *et al.*, 1985).

Assay of cAMP binding activity

Frozen cells were resuspended in Mops buffer (10 mM Mops pH 7.5 containing 10 mM benzamidine). Total extracts were pelleted at 10 000 *g* for 5 min at 4°C and the supernatants assayed for high-affinity cAMP binding activity as described (de Gunzburg *et al.*, 1984), using a final concentration of 0.1 μM [^{3}H]cAMP (40 Ci/mmol) in the assay.

Light scattering

Cells collected by centrifugation were resuspended at 10^8 cells/ml in a spectrophotometric cuvette and maintained in suspension at 23°C by gentle bubbling with oxygen as described by Gerisch and Hess (1974). cAMP was added to 0.1 μM and light scattering at 500 nm was continuously recorded.

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